VERIFICATION OF TRANSLATION

I, Toru Miura, of c/o SAKAI International Patent Office, 2-5, Kasumigaseki 3-chome, Chiyoda-ku, Tokyo 100-6019 Japan, hereby declare that I am a translator of the document attached, and attached document is a true and correct translation made by me to the best of my knowledge and belief.

Japanese Patent Application No.2001-310547, filed on October 5, 2001

Signature of Translator: _____

Toru Miura

Date : October 17, 2006

(TRANSLATION)

PATENT OFFICE
JAPANESE GOVERNMENT

This is to certify that the annexed is a true copy of the following application as filed with this Office.

DATE OF APPLICATION : October 5, 2001

APPLICATION NUMBER : Japanese Patent Application
No. 2001-310547

APPLICANT(S) : Ajinomoto Co., Inc.

Issued :

Commissioner,
Patent Office

(Official Seal)

Certificate No.

[NAME OF DOCUMENT] APPLICATION FOR PATENT
[REFERENCE NO.] 2001-233
[ADDRESS TO] COMMISSIONER, PATENT OFFICE
[INVENTOR]
[ADDRESS] c/o Ajinomoto Co., Inc.,
1-1, Suzuki-cho, Kawasaki-ku,
Kawasaki-shi, Kanagawa 210-8681 Japan
[NAME] Kenzo YOKOZEKI
[INVENTOR]
[ADDRESS] c/o Ajinomoto Co., Inc.,
1-1, Suzuki-cho, Kawasaki-ku,
Kawasaki-shi, Kanagawa 210-8681 Japan
[NAME] Sonoko SUZUKI
[INVENTOR]
[ADDRESS] c/o Ajinomoto Co., Inc.,
1-1, Suzuki-cho, Kawasaki-ku,
Kawasaki-shi, Kanagawa 210-8681 Japan
[NAME] Hiroyuki NOZAKI
[APPLICANT FOR PATENT]
[CODE NO.] 000000066
[NAME] Ajinomoto Co., Inc.
[REPRESENTATIVE] Kunio EGASHIRA
[PHONE NUMBER] 044-244-7182
[INDICATION OF FEE]
[KIND] DEPOSITED
[NUMBER] 011202
[AMOUNT OF FEE] 21000
[LIST OF ATTACHED DOCUMENTS]
[ITEM] SPECIFICATION 1
[ITEM] ABSTRACT 1

[Type of Document] Specification

[Title of the Invention] Dipeptide Producing Method, Peptide-Forming Enzyme, and Peptide-Forming Enzyme Producing Method

[Scope of Claims for Patent]

[Claim 1] A method for producing a dipeptide from an L-amino acid ester and an L-amino acid using a culture of a microbe belonging to the genus *Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium, Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora*, or *Torulopsis* that has the ability to produce a dipeptide from an L-amino acid ester and an L-amino acid, microbial cells isolated from the culture, a treated microbial product of the microbe, or a peptide-forming enzyme derived from the microbe.

[Claim 2] The method for producing a dipeptide according to claim 1, wherein an inhibitor of metalo-enzymes such as ethylenediaminetetraacetic acid (EDTA) is added to the reaction liquid when producing a dipeptide from an L-amino acid ester and an L-amino acid using a culture of a microbe having the ability to produce a

dipeptide from an L-amino acid ester and an L-amino acid, microbial cells isolated from the culture, treated microbial cells of the microbe, or a peptide-forming enzyme derived from the microbe.

[Claim 3] The method for producing a dipeptide according to claim 1 or 2, wherein the L-amino acid ester is L-alanine ester.

[Claim 4] The method for producing a dipeptide according to any one of claims 1 to 3, wherein the L-amino acid is L-glutamine or L-asparagine.

[Claim 5] A peptide-forming enzyme obtained from a microbe belonging to the genus Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium, Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora, Torulopsis, or Torulopsis and which catalyzes a reaction that produces a dipeptide from an L-amino acid ester and an L-amino acid.

[Claim 6] A method for producing a peptide-forming enzyme comprising: culturing a microbe belonging to the genus Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium;

Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora, Torulopsis, or Torulopsis in a medium, and accumulating in the culture liquid and/or cells a peptide-forming enzyme that catalyzes a reaction that produces a dipeptide from an L-amino acid ester and an L-amino acid.

[Detailed Description of the Invention]

[0001]

[Field of the Invention]

The present invention relates to a method for conveniently and economically producing a dipeptide without going through a complex synthesis method, and more particularly, to a method for producing a dipeptide from an L-amino acid ester and an L-amino acid, a peptide-forming enzyme used in the method for producing a dipeptide, and a method for producing that peptide-forming enzyme.

[0002]

[Prior Art]

Dipeptides are used in the field of pharmaceutical materials and functional foods and various fields. For example, L-alanyl-L-glutamine is used as a component of serum-free media, and is used for infusion

components since it has greater stability and higher solubility than L-glutamine.

[0003]

Chemical synthesis methods, which have been conventionally known as methods of producing dipeptides, are not necessarily simple. Known examples of such methods include a method that uses N-benzyloxycarbonylalanine (hereinafter, "Z-alanine") and protected L-glutamine (see Bull. Chem. Soc. Jpn., 34, 739 (1961), Bull. Chem. Soc. Jpn., 35, 1966 (1962)), a method that uses Z-alanine and protected L-glutamate-γ-methyl ester (see Bull. Chem. Soc. Jpn., 37, 200 (1964)), a method that uses a Z-alanine ester and unprotected glutamic acid (see Japanese Patent Application Laid-Open Publication No. H1-96194), and a method that uses a 2-substituted-propionyl halide as raw material and synthesizes an N-(2-substituted)-propionyl glutamine derivative as an intermediate (see Japanese Patent Application Laid-Open Publication No. H6-234715).

[0004]

However, in all of these methods, the introduction and elimination of a protecting group or the synthesis of an intermediate is required, so that these production methods have not been sufficiently satisfactory in view of their industrial advantages.

[0005]

Known examples of typical dipeptide production methods using enzymes include a condensation reaction using an N-protected, C-unprotected carboxy component and an N-unprotected, C-protected

amine component (Reaction 1), and a substitution reaction using an N-protected, C-protected carboxy component and an N-unprotected, C-protected amine component (Reaction 2). An example of Reaction 1 is a production method of Z-aspartylphenylalanine methyl ester from Z-aspartic acid and phenylalanine methyl ester (see Japanese Patent Application Laid-Open Publication No. S53-92729), while an example of Reaction 2 is a production method of acetylphenylalanylleucine amide from acetylphenylalanine ethyl ester and leucine amide (see Biochemical J., 163, 531 (1977)). There are extremely few examples of research reports that describe methods using N-unprotected, C-protected carboxy components. An example of a substitution reaction using an N-unprotected, C-protected carboxy component and an N-unprotected, C-protected amine component (Reaction 3) is described in Patent WO 90/01555, and example of such a reaction is a production method of arginyl leucine amide from arginine ethyl ester and leucine amide. An example of a substitution reaction using an N-unprotected, C-protected carboxy component and an N-unprotected, C-unprotected amine component (Reaction 4) is described in Patent EP 278787A, and example of such a reaction is a production method of tyrosyl alanine from tyrosine ethyl ester and alanine. Among the methods those that are able to serve as the least expensive production methods are naturally those that fall in the range of Reaction 4 involving the fewest number of protecting groups.

[0006]

However, the enzyme used in the example of the prior art of the

Reaction 4 (see Patent EP 278787A) is a comparatively expensive carboxypeptidase preparation derived from molds and plants, and the dipeptides that were produced contained amino acids that are comparatively highly hydrophobic. For the Reaction 4, there is no known method that uses an enzyme of bacterial or yeast origin, and there has been known no method for producing highly hydrophilic alanylglutamine or alanylasparagine. Under such circumstances, there has been a need for the development of an inexpensive industrial method for the production of such peptides.

[0007]

[Problems to be Solved by the Invention]

An object of the present invention is to provide a method for producing a dipeptide by an industrially advantageous and simple pathway using a starting material that can be acquired inexpensively and an enzyme source that can be supplied inexpensively (such as a microbial culture, microbial cells or a treated microbial cell product of a microbe).

[0008]

[Means to Solve the Problems]

As a result of extensive research in consideration of the aforementioned object, the inventors of the present invention have found that proline iminopeptidase has the ability to produce a peptide from an L-amino acid ester and an L-amino acid. In addition, the inventors of the present invention also have cloned and expressed the gene of the enzyme and also clearly demonstrated the broad substrate

specificity of the enzyme in peptide production using purified recombinant enzymes, thereby leading to completion of the present invention.

[0009]

The present invention provides as follows.

[0010]

(Claim 1) A method for producing a dipeptide from an L-amino acid ester and an L-amino acid using a culture of a microbe belonging to the genus *Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium, Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora*, or *Torulopsis* that has the ability to produce a dipeptide from an L-amino acid ester and an L-amino acid, microbial cells isolated from the culture, a treated microbial product of the microbe, or a peptide-forming enzyme derived from the microbe.

[0011]

(Claim 2) The method for producing a dipeptide according to claim 1, wherein an inhibitor of metalo-enzymes such as

ethylenediaminetetraacetic acid (EDTA) is added to the reaction liquid when producing a dipeptide from an L-amino acid ester and an L-amino acid using a culture of a microbe having the ability to produce a dipeptide from an L-amino acid ester and an L-amino acid, microbial cells isolated from the culture, treated microbial cells of the microbe, or a peptide-forming enzyme derived from the microbe.

[0012]

(Claim 3) The method for producing a dipeptide according to claim 1 or 2, wherein the L-amino acid ester is L-alanine ester.

[0013]

(Claim 4) The method for producing a dipeptide according to any one of claims 1 to 3, wherein the L-amino acid is L-glutamine or L-asparagine.

[0014]

(Claim 5) A peptide-forming enzyme obtained from a microbe belonging to the genus Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium, Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora, Torulopsis, or

Torulopsis and which catalyzes a reaction that produces a dipeptide from an L-amino acid ester and an L-amino acid.

[0015]

(Claim 6) A method for producing a peptide-forming enzyme comprising: culturing a microbe belonging to the genus Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium, Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora, Torulopsis, or Torulopsis in a medium, and accumulating in the culture liquid and/or cells a peptide-forming enzyme that catalyzes a reaction that produces a dipeptide from an L-amino acid ester and an L-amino acid.

[0016]

[Embodiments of the Invention]

The method for producing a dipeptide of the present invention uses a culture of a microbe having the ability to produce a dipeptide from an L-amino acid ester and an L-amino acid, microbial cells isolated from the culture, or a treated microbial product of the microbe. The reaction in the method for producing a dipeptide of the present

invention is represented by the following reaction formula:

[0017]

[Chemical 1]

$$R_1\text{-}CH(NH_2)\text{-}COOR + H_2N\text{-}CH(COOH)\text{-}R_2 \rightarrow R_1\text{-}CH(NH_2)\text{-}CONH\text{-}CH(COOH)\text{-}R_2 + ROH$$

(wherein R represents an alcohol side chain, R_1 represents the side chain of an L-amino acid ester, and R_2 represents the side chain of an L-amino acid).

[0018]

Amino acid esters are compounds that can be acquired inexpensively. The method of the present invention, in which starting materials in the form of an amino acid ester and unprotected amino acid are allowed to react in an aqueous solution using bacteria, yeast and so forth as the enzyme source, is a novel dipeptide production method not found conventionally, and is capable of inexpensively providing useful dipeptides for use in pharmaceutical materials and functional foods.

[0019]

Hereinafter, the present invention will be described in detail in the following order:

[I] Microbes having the ability to produce dipeptides from L-amino acid esters and L-amino acids

[II] Properties of peptide-forming enzyme

[III] Dipeptide production method.

[0020]

[I] Microbes Having the Ability to Produce Dipeptides from L-Amino Acid Esters and L-Amino Acids

Microbes having the ability to produce dipeptides from L-amino acid esters and L-amino acids can be used without any particular restrictions as the microbe used in the present invention. The microbes having the ability to produce dipeptides from L-amino acid esters and L-amino acids include the genus *Achromobacter, Acinetobacter, Aeromonas, Agrobacterium, Alcaligenes, Arthrobacter, Bacillus, Beijerinckia, Brevibacterium, Clavibacter, Chryseobacterium, Corynebacterium, Escherichia, Empedobacter, Enterobacter, Erwinia, Flavobacterium, Kluyvera, Microbacterium, Micrococcus, Mycoplana, Pantoea, Propionibacterium, Pseudomonas, Listonella, Rhizobium, Rhodococcus, Salmonella, Sarcina, Serratia, Stenotrophomonas, Staphylococcus, Streptomyces, Vibrio, Xanthomonas, Bullera, Candida, Cryptococcus, Filobacidium, Geotrichum, Pachysolen, Rhodosporidium, Rhodotorula, Saccharomyces, Sporoboromyces, Tremella, Torulaspora,* or *Torulopsis.* Specific examples of microbes can be given below.

[0021]

Achromobacter delmarvae	FERM BP-6988
Acinetobacter johnsonii	ATCC 9036
Aeromonas salmonicida	ATCC 14174
Agrobacterium tumefaciens	IFO 3058
Alcaligenes faecalis	ATCC 8750
Arthorobacter citreus	ATCC 11624
Bacillus subtilis	ATCC 6633

Beijerinckia indica	ATCC 9037
Brevibacterium roseum	ACTT 13825
Clavibacter michiganense	ATCC 7429
Chryseobacterium meningosepticum	ATCC 13253
Corynebacterium glutamicum	ATCC 13286
Escherichia coli	ATCC 13071
Empedobacter brevis	FERM P-18545
Enterobacter aerogenes	ATCC 13048
Erwinia amylovora	IFO 12687
Flavobacterium resinovorum	ATCC 12524
Kluyvera citrophila	FERM BP-6564
Microbacterium imperiale	ATCC 8365
Micrococcus luteus	ATCC 11880
Mycoplana bullata	ATCC 4278
Pantoea ananatis	ATCC 23822
Propionibacterium shermanii	FERM P-9737
Psudomonas putida	FERM P-18544
Listonella anguillarum	ATCC 19264
Rhizobium radiobacter	ATCC 4720
Rhodococcus rhodochrous	ATCC 21198
Salmonella typhimurium	FERM BP-6566
Sarcina lutea FERM	BP-6562
Serratia grimesii	ATCC 14460
Staphylococcus aureus	ATCC 12600
Stenotrophomonas maltophilia	ATCC 13270

Streptomyces lavendulae	ATCC 11924
Vibrio tyrogenes	FERM BP-5848
Xanthomonas maltophilia	FERM BP-5568
Bullera alba	FERM P-8032
Candida krusei	IFO 0011
Cryptococcus terreus	IFO 0727
Filobacidium capsuligenum	IFO 1119
Geotrichum amycelium	ATCC 56046
Pachysolen tannophilus	IFO 1007
Rhodosporidium diobovatum	IFO 1829
Rhodotorula minuta	IFO 0879
Saccharomyces unisporus	IFO 0724
Sporoboromyces salmonicolor	IFO 1038
Tremella foliacea	IFO 9297
Torulaspora delbrueckii	IFO 1083
Torulopsis ingeniosa	FERM P-665

[0022]

Those strains of microbes listed above that are indicated with ATCC numbers are deposited at the American Type Culture Collection (P.O. Box 1549, Manassas, VA 20110), and subcultures can be furnished by referring to each number. Those strains of microbes listed above that are indicated with IFO numbers are deposited at the Institute for Fermentation (17-85, Juso-honmachi, 2-chome, Yodogawa-ku, Osaka-shi, 532-8686, Japan), and subcultures can be furnished by referring to each number.

[0023]

Those strains of microbes listed above that are indicated with FERM numbers are deposited at the independent administrative corporation, International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology (Chuo Dai-6, 1-1 Higashi 1-Chome, Tsukuba-shi, Ibaraki-ken, 305-8566, Japan) and have been assigned a deposit number. *Achromobacter delmarvae* strain FERM BP-6988 was originally deposited on January 16, 1998, and was later transferred to international deposition on January 6, 2000. *Kluyvera citrophila* strain FERM BP-6564 was originally deposited on April 23, 1985, and was later transferred to international deposition on November 2, 1998. *Propionibacterium shermanii* strain FERM P-9737 was originally deposited on December 4, 1987. *Salmonella typhimurium* strain FERM BP-6566 was originally deposited on July 11, 1987, and was later transferred to international deposition on November 2, 1998. *Sarcina lutea* strain FERM BP-6562 was originally deposited on January 20, 1984, and was later transferred to international deposition on November 2, 1998. *Vibrio tyrogenes* strain FERM BP-5848 was originally deposited on April 25, 1983, and was later transferred to international deposition on March 4, 1997. *Xanthomonas maltophilia* strain FERM BP-5568 was originally deposited on June 14, 1995, and was later transferred to international deposition on June 14, 1996. *Bullera alba* strain FERM P-8032 was originally deposited on December 24, 1984. T*orulopsis ingeniosa* strain FERM P-665 was originally deposited on August 24, 1970.

Empedobacter brevis strain AJ-13993 and *Pseudomonas putida* strain AJ-2402 were deposited at the independent administrative corporation, International Patent Organism Depositary of the National Institute of Advanced Industrial Science and Technology on October 1, 2001. AJ-13933 has been assigned a deposit number of FERM P-18545, and AJ-2402 has been assigned a deposit number of FERM P-18544. AJ-2402 (FERM P-18544) was identified as the aforementioned *Pseudomonas putida* according to the classification experiment described below.

FERM P-18544 is a motile, aconidial rod that was identified as a bacterium belonging to the genus *Pseudomonas* based on the properties of being a Gram negative rod (0.7 to 0.8 × 1.5 to 2.0 micrometers (μm)) that forms no spore, is motile, forms round, glossy, cream-colored colonies with a completely smooth or undulating border, grows at 30°C, and is catalase positive, oxidase positive and OF test (glucose) negative. Moreover, it was identified as *Pseudomonas putida* based on the physiological properties of being nitrate reduction negative, indole production negative, acid production from glucose negative, arginine dihydrolase positive, urease negative, esculin hydrolysis negative, gelatin hydrolysis negative, ß-galactosidase negative, glucose assimilation positive, L-arabinose assimilation negative, D-mannose assimilation positive, D-mannitol assimilation positive, N-acetyl-D-glucosamine assimilation negative, maltose assimilation negative, potassium gluconate assimilation positive, n-capric acid assimilation positive, adipic acid assimilation negative,

dl-malic acid assimilation positive, sodium citrate assimilation positive, phenyl acetate assimilation positive, oxidase positive, fluorochrome production on King's B agar medium positive, levan production from sucrose positive, and weak assimilation of sorbitol.

[0024]

Wild strains or variant strains may be used for these microbes, and recombinant strains and so forth derived by cell fusion, genetic manipulation or other genetic techniques may also be used.

[0025]

To obtain microbial cells of these microbes, the microbes can be cultured and grown in a suitable medium. There is no particular restriction on the medium used for this purpose so far as it allows the microbes to grow. This medium may be an ordinary medium containing ordinary carbon sources, nitrogen sources, phosphorous sources, sulfur sources, inorganic ions, and organic nutrient sources as necessary.

[0026]

For example, any carbon source may be used so far as it can be utilized by the aforementioned microbes, and specific examples of which that can be used include sugars such as glucose, fructose, maltose, and amylose, alcohols such as sorbitol, ethanol and glycerol, organic acids such as fumaric acid, citric acid, acetic acid and propionic acid and their salts, hydrocarbons such as paraffin as well as mixtures thereof.

[0027]

Examples of nitrogen sources that can be used include ammonium salts of inorganic acids such as ammonium sulfate and ammonium chloride, ammonium salts of organic acids such as ammonium fumarate and ammonium citrate, nitrates such as sodium nitrate and potassium nitrate, organic nitrogen compounds such as peptones, yeast extract, meat extract and corn steep liquor as well as mixtures thereof.

[0028]

In addition, nutrient sources used in ordinary media, such as inorganic salts, trace metals and vitamins, can also be suitably mixed and used.

[0029]

There is no particular restriction on culturing conditions, and culturing may be carried out, for example, for about 12 to 48 hours while suitably controlling the pH and temperature to a pH range of 5 to 8 and a temperature range of 15 to 40°C under aerobic conditions.

[0030]

[III] Properties of Peptide-forming enzyme

Next, the properties of a peptide-forming enzyme purified from *Corynebacterium glutamicum* strain ATCC 13286 as an example of the aforementioned microbes.

[0031]

The peptide-forming enzyme at least has the activity to produce L-alanyl-L-glutamine using L-alanine ester and L-glutamine as substrates, and the activity to produce L-alanyl-L-asparagine using

L-alanine ester and L-asparagine as substrates.

[0032]

With respect to the enzyme action, one molecule of L-alanyl-L-glutamine and one molecule of alcohol from one molecule of L-alanine ester and one molecule of L-glutamine, and produces one molecule of L-alanyl-L-asparagine and one molecule of alcohol from one molecule of L-alanine ester and one molecule of asparagine are provided.

[0033]

The optimum pH is in the vicinity of 6.0 to 10.0, and the optimum temperature is in the vicinity of 30°C to 50°C. The molecular weight of the subunit is calculated to be 42,000 to 46,000 as determined by SDS-polyacrylamide gel electrophoresis.

[0034]

[III] Dipeptide Production Method

The dipeptide production method of the present invention produces a dipeptide from an L-amino acid ester and an L-amino acid, microbial cells isolated from the culture or treated microbial cell product of the microbe having the ability to produce a dipeptide from an L-amino acid ester and an L-amino acid.

[0035]

The peptide-forming enzyme produced by the aforementioned microbes has activity to produce a dipeptide by using an L-amino acid ester and an L-amino acid as substrates.

[0036]

As the method by which the peptide-forming enzyme produced by the aforementioned microbes is allowed to act on the L-amino acid ester and L-amino acid, the substrates may be added directly to the culture liquid while culturing the aforementioned microbes, or microbial cells may be separated from the microbial culture by centrifugation and so forth, followed by either re-suspending in buffer either directly or after washing, and then adding an L-amino acid ester and an L-amino acid and allowing them to react. Alternatively, microbial cells can be used that have been immobilized by a known method using polyacrylamide gel, carrageenan or alginic acid gel.

[0037]

In addition, crushed microbial cells, acetone-treated microbial cells or freeze-dried microbial cells may be used as the treated microbial cell product. Methods such as ultrasonic crushing, French press crushing or glass bead crushing can be used for crushing microbial cells, while methods using egg white lysozyme, peptidase treatment or a suitable combination thereof are used in the case of lysing microbial cells.

[0038]

Moreover, a peptide-forming enzyme may be recovered from the treated microbial cell product and used as a crude enzyme liquid, or the enzyme may be purified before use as necessary. Ordinary enzyme purification methods can be used for purifying the enzyme obtained from a culture. More specifically, microbial cells are collected by centrifugation and so forth, the cells are then crushed by mechanical

methods such as ultrasonic treatment, glass beads or a dynomill, and solid materials such as cell fragments are removed by centrifugation to obtain crude enzyme followed by purification of the aforementioned peptide-forming enzyme by performing ultracentrifugation fractionation, salting out, organic solvent precipitation, ion exchange chromatography, adsorption chromatography, gel filtration chromatography, hydrophobic chromatography and so forth. Note that the "peptide-forming enzyme derived from a microbe" includes not only an enzyme obtained from the treated microbial cell product by going through the aforementioned purification step, but also enzyme produced by so-called genetic engineering techniques in which the gene of the enzyme is expressed in a heterogeneous or homogeneous host.

[0039]

Namely, in the case of a fraction having activity to produce a dipeptide from an L-amino acid ester and an L-amino acid, the whole enzyme and enzyme-containing substance can be used. Here, an "enzyme-containing substance" refers to that which contains the enzyme, and includes a culture, cultured microbial cells, washed microbial cells, processed microbial cells, crude enzyme liquid and purified enzyme.

[0040]

Note that in the case of using a culture, cultured microbial cells, washed microbial cells or processed microbial cells in which the cells have been crushed or lysed, there are many cases in which an enzyme is present that decomposes the peptide produced without being

involved in peptide production, and in such cases, there are cases in which it is preferable to add a metalloprotease inhibitor like ethylenediaminetetraacetic acid (EDTA). The amount added is in the range of 0.1 mM to 100 mM, and preferably 1 mM to 50 mM.

[0041]

The amount of enzyme or enzyme-containing substance used may be enough if it is an amount in which the target effect is demonstrated (effective amount). This effective amount can be easily determined through simple, preliminary experimentation by a person with ordinary skill in the art; for example, in the case of using washed cells, the amount used is 1 to 500 g/l of reaction liquid.

[0042]

Any L-amino acid ester can be used as the L-amino acid ester so far as it is an L-amino acid ester capable of producing dipeptide with L-amino acid at the substrate specificity of the peptide-forming enzyme, and examples of such include methyl esters, ethyl esters, n-propyl esters, iso-propyl esters, n-butyl esters, iso-butyl esters and tert-butyl esters of L-amino acids. In addition, not only L-amino acid esters corresponding to naturally-occurring amino acids, but also L-amino acid esters corresponding to non-naturally-occurring amino acids or their derivatives can also be used.

[0043]

There is no particular restriction on the L-amino acid and any L-amino acid can be used so far as it produces a dipeptide with an L-amino acid ester in the substrate specificity of the peptide-forming

enzyme. L-glutamine and L-asparagine can be used preferably in the present invention.

[0044]

Each concentration of the L-amino acid ester and L-amino acid used as starting materials is 1 mM to 10 M, and preferably 0.05 M to 2 M. However, there are cases in which it is preferable to add an equimolar amount or more of L-amino acid with respect to the amount of L-amino acid ester. In addition, in the case where a high concentration of substrate inhibits the reaction, it can be adjusted to a concentration that does not cause inhibition and then successively added during the reaction.

[0045]

The reaction temperature is 3 to 70°C, and preferably 5 to 50°C, while the reaction pH is 2 to 12, and preferably 3 to 11. By carrying out the reaction in this manner for about 2 to 48 hours, a dipeptide is produced and accumulates in the reaction mixture.

[0046]

[Examples]

Hereinafter, the present invention will be described in detail by referring to the examples described below. However, the present invention is not limited to these examples. Note that in the examples, quantitative determination of L-alanine, L-alanyl-L-glutamine or L-alanyl-L-asparagine was carried out by a method using high performance liquid chromatography (column: Inertsil ODS-2 (GL Science), eluate: aqueous phosphate solution (pH 2.2, 5.0 mM sodium

1-octane sulfonate/methanol = 100/15), flow rate: 1.0 mL/min, detection: 210 nm).

[0047]

Example 1 Effect of Addition of EDTA on Production of L-Alanyl-L-Glutamine

50 mL of a medium (pH 7.0) containing 5 g of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 g of peptone in 1 L was transferred to a 500 mL Sakaguchi flask and sterilized for 15 minutes at 115°C. One loopful of of *Pseudomonas putida* strain FERM P-18544, which had been cultured for 24 hours at 30°C on an slant agar medium containing the same composition (agar: 2 g/L, pH 7.0), was inoculated into the aforementioned medium and cultured by shake culturing for 17 hours at 30°C and 120 strokes/minute. Following culturing, the microbial cells were separated by centrifugation and suspended with 100 mM borate buffer (pH 9.0) to a wet cell density of 100 g/L. 1 mL each of the cell suspension was respectively added to 1 mL of 100 mM borate buffer (pH 9.0) containing 200 mM L-alanine ethyl ester hydrochloride and 400 mM L-glutamine either in the absence of EDTA or additionally containing 20 mM EDTA (substrate solution) to bring to a final volume of 2 mL followed by allowing to react for 1 hour at 30°C. As a result, L-alanyl-L-glutamine was produced at 4.9 mM in the section added with no EDTA and at 10.1 mM in the section added with EDTA. Note that in this reaction system, under conditions in which 1 mL of 100 mM borate

buffer (pH 9.0) instead of cell suspension was added to 1 mL of substrate solution (cell-free lot) and under conditions in which 1 mL of 100 mM of borate buffer either free of EDTA or containing 20 mM EDTA but not containing L-alanine ethyl ester hydrochloride and glutamine was added to the cell suspension (substrate-free lot), production of L-alanyl-L-glutamine was not observed in either case.

[0048]

Example 2 Use of Amino Acid Ester as Substrate

1 mL of cell suspension wet cells (100 g/L) of *Pseudomonas putida* strain FERM P-18544 prepared in the same manner as Example 1 was respectively added to 1 mL of 100 mM borate buffer (pH 9.0) containing 20 mM EDTA and the following L-alanine ester hydrochlorides at 200 mM and L-glutamine at 400 mM to bring to a final volume of 2 mL, followed by allowing to react for 1 hour at 30°C. As a result, 14.9 mM L-alanyl-L-glutamine was produced in the case of using L-alanine methyl ester hydrochloride and L-glutamine as substrates, 11.4 mM L-alanyl-L-glutamine was produced in the case of using L-alanine ethyl ester hydrochloride and L-glutamine as substrates, and 0.5 mM L-alanyl-L-glutamine was produced in the case of using L-alanine-tert-butyl ester hydrochloride and L-glutamine as substrates.

[0049]

Example 3 Use of L-Amino Acid as Substrate

1 mL of a cell suspension wet cells (100 g/L) of *Pseudomonas putida* strain FERM P-18544 prepared in the same manner as Example 1 was respectively added to 1 mL of 100 mM borate buffer (pH 9.0)

containing 20 mM EDTA and the following L-alanine ester hydrochlorides at 200 mM and L-glutamine or L-asparagine at 400 mM to bring to a final volume of 2 mL, followed by allowing to react for 1 hour at 30°C. As a result, 12.7 mM L-alanyl-L-glutamine was produced in the case of using L-alanine methyl ester hydrochloride and L-glutamine as substrates, and 4.8 mM L-alanyl-L-asparagine was produced in the case of using L-alanine methyl ester hydrochloride and L-asparagine as substrates.

[0050]

Example 4 Microbes Producing L-Alanyl-L-Glutamine

50 mL of a medium (pH 7.0) containing 5 g of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate, 10 g of yeast extract and 10 of peptone in 1 L was transferred to a 500 mL Sakaguchi flask and sterilized for 15 minutes at 115°C. One loopful of of each of the bacteria shown in Table 1, which had been cultured for 24 hours at 30°C on an slant agar medium (agar: 2 g/L, pH 7.0) containing 5 g of glucose, 10 g of yeast extract, 10 g of peptone and 5 g of NaCl, was inoculated into the aforementioned medium and cultured by shake culturing for 17 hours at 30°C and 120 strokes/minute. After the culturing, the microbial cells were separated by centrifugation and suspended with 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells. 50 mL of a medium (pH 6.0) containing 5 g of glucose, 5 g of ammonium sulfate, 1 g of monopotassium phosphate, 3 g of dipotassium phosphate, 0.5 g of magnesium sulfate,

5 g of yeast extract, 5 g of malt extract, and 10 of peptone in 1 L was transferred to a 500 mL Sakaguchi flask and sterilized for 15 minutes at 115°C. One loopful of of each of the bacteria shown in Table 1, which had been cultured for 24 hours at 30°C on an slant agar medium (agar: 2 g/L, pH 6.0) containing 5 g of glucose, 10 g of yeast extract, 5 g of melt extract, 10 g of peptone and 5 g of NaCl, was inoculated into the aforementioned medium and cultured by shake culturing for 17 hours at 25°C and 120 strokes/minute. After the culturing, the microbial cells were separated by centrifugation and suspended with 0.1 M borate buffer (pH 9.0) containing 10 mM EDTA to 100 g/L as wet microbial cells. 0.1 mL of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA, 200 mM L-alanine methyl ester hydrochloride and 400 mM L-glutamine was respectively added to 0.1 mL of these bacterial and yeasty cell suspensions to bring to a final volume of 0.2 mL followed by allowing to react for 2 hours at 25°C. The amounts (mM) of L-alanyl-L-glutamine (Ala-Gln) produced at this time are shown in Table 1 and Table 2.

[0051]

[Table 1]

Microbe	Ala-Gln (mM)
Achromobacter delmarvae FERM BP-6988	4.2
Acinetobacter johnsonii ATCC 9036	3.8
Aeromonas salmonicida ATTCC 14174	1.8
Agrobacterium tumefaciens IFO 3058	8.2
Alcaligenes faecalis ATCC 8750	6.3
Arthrobacter citreus ATCC 11624	2.7
Bacillus subtilis ATCC 6633	1.1
Beijerinckia indica ATCC 9037	13.0
Brevibacterium roseum ATCC 13825	2.6
Clavibacter michiganense ATCC 7429	1.9
Chryseobacterium meningosepticum ATCC 13253	3.2
Corynebacterium glutamicum ATCC 13286	7.2
Escherichia coli ATCC 13071	1.0
Empedobacter brevis FERM P-18545	45.0
Enterobacter aerogenes ATCC 13048	0.8
Erwinia amylovora IFO 12687	0.9
Flavobacterium resinovorum ATCC 12524	3.8
Kluyvera citrophila FERM BP-6564	3.1
Microbacterium imperiale ATCC 8365	4.3
Micrococcus luteus ATCC 11880	0.9
Mycoplana bullata ATCC 11880	7.1
Pantoea ananatis 23822	0.7
Propionibacterium shermanii FERM P-9737	2.9
Pseudomonas putida FERM BP-8101	14.8

[Table 2]

Microbe	Ala-Gln (mM)
Listonella angulillarum ATCC 19264	2.9
Rhizobium radiobacter ATCC 4720	10.2
Ehodococcus rhodochrous ATCC 21198	7.0
Salmonella typhimurium FERM BP-6566	1.6
Sarcina lutea FERM BP-6562	1.9
Serratia grimesii ATCC 14460	0.8
Stenotrophomonas maltophilia ATCC 13270	1.2
Staphylococcus aureus ATCC 12600	0.7
Streptomyces lavendulae ATCC 11924	5.1
Vibrio tyrogenes FERM BP-5848	30.0
Xanthomonas maltophilia FRRM BP-5668	9.8
Bullera alba FERM P-8032	1.8
Candida krusei IFO 0011	1.3
Cryptococcus terreus IFO 0727	2.9
Filobacidium capsuligenum IFO 1119	0.6
Geotrichum amycelium ATCC 56046	10.6
Pachysolen tannophilus IFO 1007	1.9
Rhodosporidium diobovatum IFO 1829	4.8
Rhodotorula minuta IFO 0879	3.9
Saccharomyces unisporus IFO 0724	4.6
Sporoboromyces salmonicolor IFO 1038	5.2
Tremella foliacea IFO 9297	1.9
Torulaspora delbrueckii IFO 1083	1.8
Torulopsis ingeniosa FERM P-665	2.1

[0052]

Example 5 Effect of Temperature on Production of L-Alanyl-L-Glutamine

1 mL of the suspension of *Pseudomonas putida* strain FERM P-18544 cells (100 g/L) prepared in accordance with the bacterial culturing method of Example 4 was respectively added to 1 mL of 100 mM borate buffer (pH 9.0) containing 10 mM EDTA, 200 mM L-alanine methyl ester hydrochloride and 400 mM L-glutamine to bring to a final volume of 2 mL, followed by allowing to react for 1 hour at temperatures of 20°C, 30°C and 40°C, respectively. Those results are shown in

Table 3. Production of L-alanyl-L-glutamine (Ala-Gln) demonstrated the highest value at a temperature of 40°C in the case of *Pseudomonas putida* strain FERM P-18544. In the meanwhile, it demonstrated the highest value at a temperature of 20°C in the case of *Pseudomonas putida* strain FERM P-18545.

[0053]

[Table 3]

Microbe	Ala-Gln Produced (mM)		
	20°C	30°C	40°C
Pseudomonas putida FERM P-18544	8.2	16.9	20.8
Empedobacter brevis FERM P-18545	57.9	23.6	6.5

[0054]

Example 6 Purification of Peptide-forming enzyme from *Corynebacterium glutamicum* strain ATCC 13286 and Production of L-Alanyl-L-Glutamine by the Purified Enzyme

500 mL of a medium containing 5 g of glycerol, 5 g of yeast extract, 5 g of peptone, 5 g of NaCl and 5 g of L-alanine amide hydrochloride in 1 L was transferred to a 5 L Sakaguchi flask and sterilized for 20 minutes at 120°C. Culture liquid of *Corynebacterium glutamicum* strain ATCC 13286 cultured for 20 hours in medium of the same composition as above was inoculated into the medium to be 5% (v/v), and cultured for 20 hours at 30°C and 120 strokes/minute. Microbial cells were collected from 8 L of this culture liquid by centrifugation. The subsequent procedure was carried out either on

ice or at 4°C. After washing the microbial cells with 50 mM potassium phosphate buffer (pH 7.0), the cells were subjected to crushing treatment for about 10 minutes using glass beads having a diameter of 0.1 millimeter. The glass beads and crushed cell liquid were then separated, and the crushed cell fragments were removed by centrifugation for 30 minutes at 20,000×gravity (g) to obtain a cell-free extract. Moreover, the insoluble fraction was removed by ultracentrifugation for 60 minutes at 200,000×g to obtain a soluble fraction in the form of the supernatant. Ammonium sulfate was then added to the resulting soluble fraction to 60% saturation followed by recovery of the precipitate by centrifuging for 30 minutes at 20,000×g. The resulting precipitate was dissolved in a small amount of 50 mM potassium phosphate buffer (pH 7.0) and then dialyzed against 50 mM potassium phosphate buffer (pH 7.0). This enzyme liquid was then applied to a Q-Sepharose HP column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), and the enzyme was eluted over a linear concentration gradient of 50 mM potassium phosphate buffer (pH 7.0) containing 0 to 1.0 M sodium chloride. The active fraction was collected and applied to a Superdex 200 pg column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), and the enzyme was then eluted with the same buffer. The active fraction was collected and dialyzed against 20 mM potassium phosphate buffer (pH 7.0) containing 0.5 M ammonium sulfate, and then applied to a Phenyl-Sepharose HP column pre-equilibrated with 20 mM potassium phosphate buffer (pH 7.0) containing 0.5 M ammonium sulfate. The

enzyme was then eluted over a linear concentration gradient of 20 mM potassium phosphate buffer (pH 7.0) containing 0.5 to 0 M ammonium sulfate. The active fraction was collected and dialyzed against 50 mM potassium phosphate buffer (pH 7.0), and this was then applied to a MonoQ column pre-equilibrated with 50 mM potassium phosphate buffer (pH 7.0), after which enzyme was eluted over a linear concentration gradient of 50 mM potassium phosphate buffer (pH 7.0) containing 0 to 1.0 M sodium chloride. The purified peptide-forming enzyme was uniformly purified on the basis of electrophoresis in this manner. The specific activity of the purified enzyme was 9.841 U/mg, and the specific activity of the purified peptide-forming enzyme was increased about 246-fold as a result of going through these purification steps. In addition, as a result of applying the molecular weight of the purified enzyme standard to SDS polyacrylamide electrophoresis, a uniform band was detected at the position calculated to represent a molecular weight of 42,000 to 46,000. Measurement of enzyme titer was carried out as described below. 200 μM of Tris-HCl buffer (pH 9.0), 50 μM of L-alanine amide and a suitable amount of enzyme liquid were added and mixed to a final volume 1 mL, and after allowing to react for 60 minutes at 30°C, 4 mL of aqueous phosphoric acid (pH 2.1) was added to stop the reaction. The alanine produced was quantified by high-performance liquid chromatography, and the amount of enzyme that produces 1 μM of L-alanine in 1 minute was defined as 1 unit.

This purified enzyme was then added to borate buffer (pH 9.0) containing EDTA, L-alanine methyl ester hydrochloride and L-glutamine

(or L-asparagine), mixed to the total volume of 1 mL (for the final concentrations, the amount of enzyme added was 2 units as alanine amide decomposition activity, EDTA was at 10 mM, L-alanine methyl ester hydrochloride was at 100 mM and L-glutamine (or L-asparagine) was at 200 mM borate buffer at 100 mM), and allowed to react for 4 hours at 30°C. (Note that the number of units of enzyme does not indicate the production activity with respect to producing alanylglutamine from alanine methyl ester and glutamine, but rather simply indicates alanine amide decomposition activity.) The amount of L-alanyl-L-glutamine produced at this time was 50.2 mM, while the amount of alanylasparagine produced was 49.8 mM.

[0055]

[Effects due to the Invention]

According to the dipeptide producing method of the present invention, a dipeptide can be produced using an L-amino acid ester and L-amino acid which can be acquired inexpensively without going through a complex synthesis method, making it possible to reduce the production cost of dipeptides useful as pharmaceutical materials, functional foods, and the like.

[Type of Document] Abstract

[Abstract]

[Object] To provide a method for producing dipeptide using inexpensively acquirable starting materials by an industrially advantageous and simple pathway.

[Means] Dipeptide is produced from L-amino acid ester and L-amino acid using a culture of microbes having the ability to produce a dipeptide from an L-amino acid ester and an L-amino acid, using microbial cells isolated from the culture, or a treated microbial cell product of the microbe.

[Selected Figure] None